Mitogenic function of human procathepsin D: the role of the propeptide

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The proform of cathepsin D is secreted by some human breast-cancer cell lines upon stimulation with oestrogen. In these cell lines, procathepsin D was described to act as an autocrine mitogen, and a correlation between the cathepsin D concentration in tumour tissues and poor prognosis for the patient was demonstrated in several independent investigations. In the present study, we focused on the mechanism of procathepsin D mitogenic activity. Procathepsin D isolated from secretions of ZR-75-1 breast-cancer cell line was used to test for mitogenic activity on a set of seven human cell lines. For nanomolar procathepsin D concentrations, we found a stronger dose-responsive cellular reaction in the case of several different human breast-cancer-derived cell lines. The mitogenic activity was not blocked by the inhibition of proteolytic activity nor by the inhibition of the interaction of procathepsin D with mannose-6-phosphate receptors. On the other hand, the addition of antibodies raised against the propeptide impaired the mitogenic activity of procathepsin D, and a synthetic peptide alone corresponding to the propeptide of procathepsin D produced similar effects, as did the zymogen molecule. The synthetic propeptide was shown to block partially the interaction of procathepsin D with the cellular surface. Our results indicate that the mitogenic function involves the propeptide of cathepsin D, which appears to be recognized by a surface receptor.

INTRODUCTION

Cathepsin D (CD) (EC 3.4.23.5) is an aspartic protease found in lysosomes of all mammalian cells, and is considered to be one of the main catabolic proteinases [1]. It is, in the form of zymogen, targeted via the mannose 6-phosphate (M6P) pathway (reviewed by Kornfeld and Mellman [2]). The two M6P receptors involved in the lysosomal targeting of procathepsin D (pCD) are localized both intracellularly and on the outer cell membrane. Two roles were identified for the cation-independent M6P receptor present on the cellular surface. The receptor recognizes molecules which contain the M6P tag and recaptures them [3], and it also binds and mediates the effect of the insulin-like growth factor II (IGF II) [4].

pCD is secreted from cultured human cell lines at low levels but in response to oestrogen stimulation, pCD becomes the major secreted protein in several human breast-cancer cell lines [5–8]. Many clinical studies have revealed the correlation between the total CD concentration in the tumour and high risk for the patient [7,9–11]. Studies of the pCD secretion from human breast-cancer cell lines have shown that pCD acts as an autocrine mitogen [7]. Physiological relevance of this function was also proposed [8] but the properties of pCD involved in the mitogenic function are not known.

In our study, we have investigated the mechanism of the mitogenic function of pCD and compared it with the mitogenic function of the IGF II. The mitogenic function was tested by incubation of cells with pCD isolated from secretions of ZR-75-1 cell line, with commercially available IGF II, and with a synthetic peptide corresponding to the pCD propeptide sequence. The cellular response was measured by monitoring of the cellular proliferation of different human cell lines. pCD exhibited a mitogenic effect on cell lines derived from breast-cancer tissues. Our data indicate that this mitogenic effect is mediated by the

structure within the propeptide of pCD, and does not involve pCD interaction via the M6P group nor CD proteolytic activity. Finally our data strongly suggest that on cells derived from breast cancer there is present a cell-surface receptor which is able to recognize the propeptide of pCD.

MATERIALS AND METHODS

Materials

N-Glycanase (EC 3.5.1.53) was a product of Genzyme (Cambridge, MA, U.S.A.), pig pepsinogen A (PPGN; EC 3.4.23.1) was purchased from Worthington (Freeholf, NJ, U.S.A.), human cathepsin D (EC 3.4.23.5) was obtained from Biodesign (Kennebunk, ME, U.S.A.), human IGF II, bovine cathepsin D (BCD), bovine haemoglobin, RPMI 1640 and Iscove's modified Dulbecco's medium, *β*-oestradiol, Hepes, propidium iodide, 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyltetrazolium bromide (MTT) and fluorescein isothiocyanate (FITC) were purchased from Sigma (Sigma, St. Louis, MO, U.S.A.). Ficoll-Hypaque and Fetal Clone were from Hyclone Laboratories (Logan, UT, U.S.A.), pepstatin A from Serva (Heidelberg, Germany) and Protein A–Sepharose from Pharmacia (Uppsala, Sweden). Other chemicals were of the highest purity available.

Antibodies

Anti-pCD IgG antibodies (anti-pCD) were raised against a synthetic propeptide of pCD, and their specificity for zymogen of CD only was documented earlier [12]. The anti-BCD IgG antibodies were kindly provided by Dr. J. Tang, Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A. and their recognition of both human mature CD and pCD was demonstrated elsewhere [12].
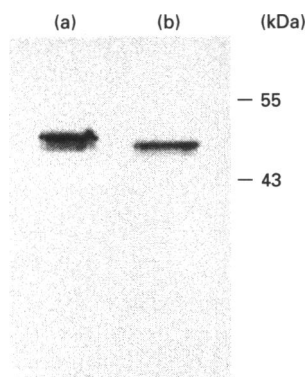

Figure 1   Western blot of pCD before and after deglycosylation

Procathepsin D before (a) and after deglycosylation (b) analysed by SDS/PAGE, blotted, and visualized using pig anti-pCD IgG antibodies and rabbit anti-(pig IgG) antibodies conjugated with peroxidase.

Procathepsin D

pCD was isolated from pooled media of cell line ZR-75-1 cells treated with 10 nM β-oestrogen as described previously [13]. Briefly, a two-step procedure was employed using immunoaffinity chromatography based on anti-pCD antibodies conjugated to Protein A–Sepharose, followed by two anion-exchange Mono Q f.p.l.c. separations at pH 7.2. The isolated pCD was without contaminants as judged by SDS/PAGE, identification by antibodies and inhibition of the activity by pepstatin A [13]. As an independent test of purity of our preparation, we tested solutions of pCD with the anti-CD antibodies conjugated to Protein A–Sepharose. An aliquot (1 ml) of the pCD solution (100 ng/ml in PBS) was incubated with 100 μl of slurry of anti-CD antibodies attached to the Protein A–Sepharose (10 μg of the anti-CD antibody was preincubated with Protein A–Sepharose and the gel was washed several times with PBS before addition to the solution of pCD) at 4 °C for 4 h. The mixture was centrifuged and the supernatant tested for biological activity. Such solutions lost both proteolytic activity at low pH and the mitogenic activity. When pCD solution was preincubated with Protein A–Sepharose only (without the addition of anti-CD antibodies) no loss of activity was observed.

Propeptide

The synthesis of the 44-amino-acid-long peptide corresponding to the propeptide of pCD has been described previously [14].

Cell lines

Human cell lines U937 (monocytoid) and Raji (B lympho-blastoid) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, U.S.A.). The cell line 8402 (T lymphoblastoid) was obtained from The Tissue Culture Facility of the Lineberger Cancer Research Center of the University of North Carolina at Chapel Hill, Chapel Hill, NC, U.S.A. Human breast-cancer cell lines ZR-75-1, MDA-MB-483, MDA-MB-436 and MDA-MB-231 were obtained from Dr. R. Ceriani of the John Muir Cancer and Aging Research Institute, Walnut Creek, CA, U.S.A. The cancer cell lines were grown in RPMI 1640 medium with Hepes buffer supplemented with 10 % (v/v) heat-

inactivated Fetal Clone, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a 5 % CO_2/95 % air incubator. All other cell lines were incubated in the same way, except that the Fetal Clone was supplemented with fetal-calf serum (FCS). Steroid-deprived cells were used in all experiments and the levels of oestrogens in FCS were $\leqslant$ 1 pg/ml. The concentrations of pCD in medium before growth, as well as in medium after growth of all types of human cells without potentiation with oestrogen, were below 1 pM, as measured by the proteolytic activity of pCD (results not shown).

pCD modifications

For treatment with N-glycanase, 1 μg of pCD in 50 mM Tris/HCl buffer, pH 7.5, was treated with 1 unit of N-glycanase for 16 h at 37 °C. The cleavage of oligosaccharides was confirmed by altered mobility on SDS/PAGE (Figure 1). The electrophoresis and Western blot were done as described earlier with a stacking gel containing 4 % (w/v) acrylamide and a separating gel of 14 % (w/v) acrylamide [12]. For the FITC labelling we used the following procedure: 1 ml of pCD (concentration 100 μg/1 ml) was dialysed four times (12 h each step) against 250 ml of 100 mM borate buffer, pH 8.5. A sample (20 μl) of FITC solution (25 mg dissolved in 1 ml of dimethylsulphoxide) was added to the dialysed solution of pCD and incubated for 2 h at room temperature. The solution was the dialysed (using dialysis tubing with a nominal cutoff of 30 kDa) against five changes (250 ml, 12 h each step) of 50 mM Tris/HCl buffer, pH 7.2.

Cellular proliferation

Cells were first incubated for 2 days in 0.1 % FCS and then were washed six times in Iscove's modified Dulbecco's medium and seeded in 96-well tissue-culture plates at a density of 5×10^4 cells/ml (150 μl/well) with or without different amounts and types of additives. After 7 days, 20 μl of MTT (5 mg/ml in PBS) was added to each well and the plates were cultivated for an additional 4 h. The incorporation of MTT was stopped by the addition of 50 μl of 10 % (w/v) SDS in 0.01 M HCl and the absorbance at 570 nm was measured 24 h later using a Microplate Reader MR600 (Dynatech, Alexandria, VA, U.S.A.).

Staining of cell with FITC–pCD and flow-cytometry analysis

The cells were harvested by gently scratching using cell scrapers and were then resuspended in PBS. MDA-MB-231 cells (5×10^5) were incubated with 50 ng of FITC–pCD for 30 min at 4 °C. For the inhibition studies, the cells were first incubated with 10 ng of the propeptide. When the FITC-labelled anti-pCD were used, the cells were first incubated for 30 min with pCD and then incubated with the FITC-labelled anti-pCD antibodies. In all experiments the cells were, after preincubations, washed once by centrifugation through a 3 ml cushion of 12 % (w/v) BSA in PBS containing 10 mM sodium azide. After removing the supernatant, the cells were resuspended in 0.2 ml of PBS. Flow cytometry was performed with an EPICS Profile II (Coulter Electronics Inc., Miami Lakes, FL, U.S.A), and the data obtained from at least 10^4 cells in each sample were stored in list mode. The binding of labelled pCD to the cells, gated by light scatter, was assessed by analysing the stored list mode data with the Epics Elite Flow Cytometry Workstation software (Coulter).

Sterile buffers and aseptic conditions were used at all steps. All media and buffers were tested for endotoxin contaminations, and shown to contain less than 0.1 ng/ml of endotoxin using the *Limulus* lysate test (E-TOXATE, Sigma).

Table 1   Influence of the addition of pCD and IGF II with different additives on the growth of seven human cell lines

The results from growth in a control medium supplemented with FCS or with no additives are given for comparison. The concentrations were: pCD, 0.4 nM (20 ng/ml); IGF II, 2.7 nM (20 ng/ml); M6P, 10 mM; pepstatin A, 1 μM; deglycosylated pCD (Deglyc.pCD), 0.4 nM. See the Materials and methods section for details. The results in these experiments and in subsequent proliferation experiments represent the mean $\pm$ S.D. of five experiments. The additions of chemicals used for the deglycosylation or the addition of pepstatin A to the cell (with no FCS, pCD or IGF II) had no effect on the cellular proliferation. The values of A_{570} which are significantly elevated to compare with the control experiment are typed in bold.

Conditions	Cell proliferation (MTT Assay; A_{570})						
	U937	8402	Raji	ZR-75-1	MDA-MB-483	MDA-MB-231	MDA-MB-436
No addition	0.11 ± 0.01	0.09 ± 0.01	0.12 ± 0.06	0.12 ± 0.02	0.08 ± 0.01	0.19 ± 0.05	0.13 ± 0.04
FCS	**1.26 ± 0.25**	**1.07 ± 0.17**	**0.92 ± 0.15**	**0.73 ± 0.18**	**0.87 ± 0.09**	**1.06 ± 0.19**	**0.80 ± 0.11**
pCD	0.23 ± 0.03	0.19 ± 0.04	0.28 ± 0.07	**0.53 ± 0.07**	**0.62 ± 0.07**	**0.61 ± 0.03**	**0.53 ± 0.04**
pCD + pepstatin A	0.17 ± 0.04	0.18 ± 0.05	0.30 ± 0.07	**0.47 ± 0.09**	**0.53 ± 0.12**	**0.60 ± 0.11**	**0.69 ± 0.19**
pCD + M6P	0.18 ± 0.03	0.15 ± 0.02	0.15 ± 0.05	**0.55 ± 0.11**	**0.65 ± 0.14**	**0.58 ± 0.09**	**0.62 ± 0.11**
Deglyc. pCD	0.09 ± 0.03	0.26 ± 0.09	0.19 ± 0.06	**0.51 ± 0.11**	**0.61 ± 0.10**	**0.47 ± 0.10**	**0.36 ± 0.05**
IGF II	**0.95 ± 0.18**	**0.79 ± 0.13**	**0.99 ± 0.15**	**0.53 ± 0.09**	**0.53 ± 0.08**	**0.64 ± 0.12**	**0.65 ± 0.10**
IGFII + M6P	0.16 ± 0.10	0.21 ± 0.04	0.22 ± 0.05	0.12 ± 0.06	0.15 ± 0.03	0.20 ± 0.03	0.12 ± 0.02

Table 2   Influence of two types of antibodies on the pCD-potentiated proliferation of seven human cell lines and the influence of propeptide addition

The antibodies used were anti-pCD or anti-CD and their concentrations were 5 μg/ml of the IgG fraction. Concentrations used: CD, 2 nM; human CD, 2 nM; the propeptide of pCD, 20 nM; and PPGN, 20 nM. Other conditions were the same as described in Table 1.

Conditions	Cell proliferation (MTT assay; A_{570})						
	U937	8402	Raji	ZR-75-1	MDA-MB-483	MDA-MB-231	MDA-MB-436
No addition	0.11 ± 0.01	0.09 ± 0.01	0.12 ± 0.06	0.12 ± 0.02	0.08 ± 0.01	0.19 ± 0.05	0.13 ± 0.04
FCS	**1.26 ± 0.25**	**1.07 ± 0.17**	**0.92 ± 0.15**	**0.73 ± 0.18**	**0.87 ± 0.09**	**1.06 ± 0.19**	**0.80 ± 0.11**
pCD	0.23 ± 0.03	0.19 ± 0.04	0.28 ± 0.07	**0.53 ± 0.07**	**0.62 ± 0.07**	**0.61 ± 0.03**	**0.53 ± 0.04**
pCD + anti-CD	0.23 ± 0.05	0.21 ± 0.03	0.22 ± 0.06	**0.61 ± 0.10**	**0.66 ± 0.12**	**0.59 ± 0.11**	**0.57 ± 0.12**
pCD + anti-pCD	0.19 ± 0.04	0.15 ± 0.03	0.09 ± 0.04	0.21 ± 0.07	0.22 ± 0.06	0.17 ± 0.05	0.18 ± 0.05
IGF II	**0.95 ± 0.18**	**0.79 ± 0.13**	**0.99 ± 0.15**	**0.53 ± 0.09**	**0.53 ± 0.08**	**0.64 ± 0.12**	0.65 ± 0.10
IGF II + anti-pCD	**0.88 ± 0.22**	**0.81 ± 0.20**	**1.17 ± 0.30**	**0.60 ± 0.11**	**0.65 ± 0.12**	**0.60 ± 0.09**	**0.61 ± 0.21**
Human CD	0.15 ± 0.03	0.20 ± 0.05	0.17 ± 0.05	0.09 ± 0.02	0.11 ± 0.05	0.15 ± 0.07	0.14 ± 0.03
BCD	0.19 ± 0.04	0.19 ± 0.06	0.26 ± 0.07	0.15 ± 0.04	0.12 ± 0.04	0.19 ± 0.05	0.13 ± 0.03
PPGN	0.14 ± 0.02	0.15 ± 0.07	0.18 ± 0.08	0.11 ± 0.02	0.12 ± 0.06	0.11 ± 0.02	0.09 ± 0.03
Propeptide	0.22 ± 0.04	0.14 ± 0.07	0.22 ± 0.06	**0.42 ± 0.07**	**0.50 ± 0.11**	**0.48 ± 0.09**	**0.49 ± 0.07**
Propeptide + anti-CD	0.20 ± 0.03	0.17 ± 0.05	0.16 ± 0.05	**0.44 ± 0.05**	**0.52 ± 0.09**	**0.52 ± 0.11**	**0.45 ± 0.09**
Propeptide + anti-pCD	0.11 ± 0.04	0.11 ± 0.06	0.15 ± 0.04	0.15 ± 0.07	0.16 ± 0.06	0.15 ± 0.04	0.18 ± 0.04

RESULTS

Influence of pCD, IGF II, and pCD derivatives on the proliferation of human cell lines

The effects of the pCD and IGF II on the proliferation of cell lines ZR-75-1, MDA-MB-483, MDA-MB-231, MDA-MB-436, Raji, U937 and 8402 are summarized in Table 1. The proliferation was measured by the incorporation of MTT by cells. The FCS- or IGF II-supplemented media were used as positive controls. The proliferation of breast-cancer cell lines was increased in the same way for both pCD and IGF II, while the cell lines which are not derived from breast-cancer tissues responded to pCD much less than to IGF II. The proliferative activity of pCD and IGF II for breast-cancer cell lines was dose-responsive, with maximum proliferative activity at concentrations of both molecules of 20 ng/ml as described earlier [15]. In subsequent proliferative experiments, the concentrations of pCD and IGF II were 20 ng/ml (0.4 nM for pCD and 2.7 nM for IGF II). This experimental approach was used to study the mechanism of the mitogenic effect exhibited by pCD.

The influence of the addition of pepstatin A and M6P, together with the effect of deglycosylation of pCD, is presented in Table

1. Pepstatin A is a strong inhibitor of CD, with K_i at the picomolar level [16], and was used to test the involvement of the proteolytic activity of CD on the observed mitogenic function. The hypothetical role of the M6P residues of pCD was investigated by either the addition of M6P or by the deglycosylation of pCD.

These experiments showed that the addition of pepstatin A, M6P, or deglycosylation of pCD had very little influence on the observed proliferative activity of pCD. Nevertheless, high concentrations of M6P had a strong inhibitory effect on IGF II proliferative activity, showing that strong interaction of M6P with the cation-independent M6P receptor occurs (reviewed by von Figura [17]). These results indicated that the mitogenic function of pCD does not depend on the proteolytic activity nor on the presence of the M6P tag.

A strong inhibition of the mitogenic function was observed when antibodies raised against the propeptide of pCD were used (Table 2). The specificity of two antibodies used in this experiment has been described previously [12]; briefly, the anti-pCD antibodies recognize epitopes contained within the propeptide of pCD only, while anti-CD antibodies interact with epitopes of the mature enzyme and not with the propeptide. The inhibition
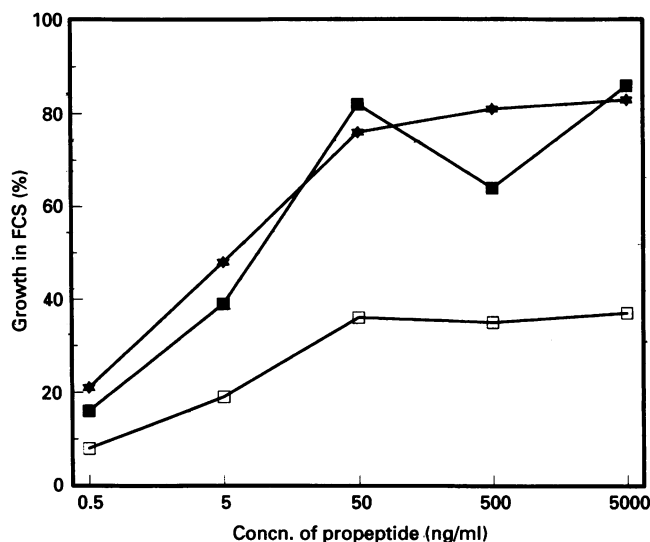
Figure 2   The dose–response curves of the proliferative effect of the propeptide for human breast-cancer cell lines MDA-MB-231 (□), MDA-MB-436 (✳) and ZR-75-1 (■)

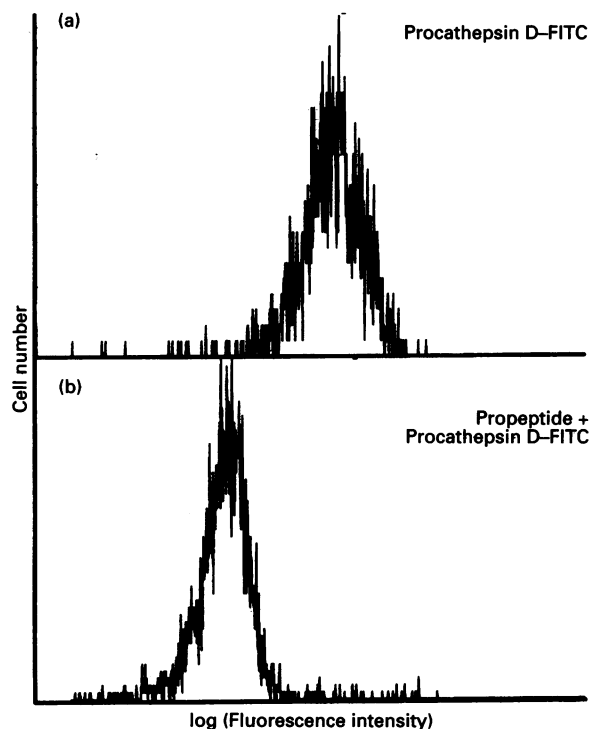**Figure 3   Interaction of FITC-labelled pCD with MDA-MB-231 cells**

FITC-labelled pCD (50 ng) was incubated with 5×10^5 cells in 100 μl for 30 min (a), or with the same amount of cells preincubated with 10 ng of the propeptide (b), and analysed by flow cytometry. An 85% decrease in the intensity of the fluorescence signal [shift to the lower values of log (Fluorescence)] was observed when the activation peptide was added.

of the mitogenic effect observed for anti-pCD antibodies was specific for pCD, and these antibodies had no effect on the function of IGF II. As an additional control, we used the anti-CD antibodies bound to Protein A–Sepharose to remove the

pCD from solutions before assaying the mitogenic activity. No activity was detected in these controls. These experiments confirmed the purity of our preparation, and supported the idea that the anti-pCD antibodies were specifically blocking the mitogenic activity of pCD. This result suggested the importance of the propeptide of pCD in its mitogenic functions.

To investigate further the role of the propeptide in the mitogenic function we used a synthetic peptide corresponding to the propeptide of pCD, and similar proliferative activity to that of pCD was observed (Table 2). The breast-cancer-derived cell lines responded in the same way to pCD and the synthetic propeptide, while cell lines which did not react with pCD did not show any response to the peptide alone. A dose–response curve for the synthetic peptide is shown on Figure 2. No proliferative activity was observed for control molecules i.e. PPGN or mature cathepsin D (Table 2). The PPGN control experiment was included as a protein of similar overall structure. Mature pepsin has a similar three-dimensional structure to CD [18]; however, the sequences and the three-dimensional structures of the propeptides of both enzymes differ substantially in the segment 43P-5 of pCD [13].

We have also examined the influence of the addition of antibodies against CD on the mitogenic function of the propeptide of pCD. Results were similar to those obtained for pCD. The function of the propertide was blocked by anti-pCD antibodies but not by the anti-CD antibodies (Table 2). These results confirmed the hypothesis that the propeptide of pCD plays an important role in its mitogenic activity.

Interactions of FITC-labelled pCD with MDA-MB-231 cells

To demonstrate the interaction of pCD with the surface of human cells, we used pCD conjugated to a fluorescent marker (FITC) (Figure 3). pCD–FITC conjugate labelled both tested cell lines ZR-75-1 and MDA-MB-231 (results shown for MDA-MB-231 cells only). Figure 3 also shows that the fluorescent labelling of the cell line with pCD conjugated to FITC is inhibited at 85 % by preincubation of the cells with the synthetic propeptide. Similar blocking was found for cells preincubated with non-labelled pCD (results not shown). In additional control experiments, we first incubated the FITC–pCD conjugate with anti-pCD antibodies, and no labelling of cells was observed.

When non-labelled pCD was preincubated with cells and then FITC-labelled anti-pCD antibodies were added, no staining was detected (results not shown). We have not detected any significant staining of U937 cells by the pCD–FITC conjugate. Both these experiments support the importance of the propeptide in the pCD–cellular surface interactions.

DISCUSSION

In this study we have shown that the mechanism of the mitogenic function of pCD is a function of the propeptide, rather than a result of the proteolytic activity of activated extracellular CD, or of M6P residues of pCD carbohydrate groups binding to the M6P receptor. Our earlier experiments had revealed a pronounced activity of pCD in promoting the proliferation of a set of human breast-cancer cell lines [15]. A dose–response dependence, with saturation of the signal, was observed, indicating an interaction limited by the number of responding molecules. To determine the mechanism of these reactions, we examined three features of pCD which might be expected to be involved: (i) proteolytic activity, (ii) presence of M6P residues, and (iii) existence of the propeptide. Throughout the study we used the addition of IGF II as a positive control for the interaction and

activation of the cation-independent M6P receptor because this receptor both transmits the signal of the IGF II and interacts with M6P residues present on the pCD structure [19].

Proteolytic activity

The activation of pCD is accomplished by the removal of the 44 amino acid propeptide at the N-terminus of the proenzyme, and takes place at the low pH of lysosomes (reviewed by Hasilik [20]). The activation is achieved by a combination of limited auto-proteolysis and cleavage by other lysosomal proteinases. CD is a proteinase with a pH optimum close to 3, and its activity rapidly falls at pH values above 5 [21]. Proteolytic activity of pCD in the extracellular space has not yet been reported. Nevertheless, tissues with high consumption of energy, as in the case of tumour tissues, may locally produce low pH [22], and consequently allow the activation of a secreted pCD. In our experiments no inhibition of the mitogenic activity of pCD by pepstatin A (a strong inhibitor of aspartic proteinases) was observed. When mature, active CD was used in our experiments, no mitogenic effect was detected. Both results excluded the involvement of CD proteolytic activity in pCD mitogenic functions under our experimental conditions.

M6P residues

High concentrations of M6P inhibit the interaction of the pCD with cell-surface M6P receptors and block pCD internalization [23]. If pCD mitogenic activity was realized through the M6P residues, then the addition of M6P would inhibit it. In our experiments the addition of M6P had no influence on mitogenic activity of pCD. Removing the sugar moiety from the pCD structure slightly lowered the mitogenic activity of pCD in both proliferative and activation experiments, which can be explained by the fact that the deglycosylation of enzymes is often accompanied by some degree of disturbance of the original protein structure. Overall the data show that the M6P sugar structures are not crucial for the mitogenic activity of pCD.

Propeptide

In mammalian cells there are at least two mechanisms known for targeting pCD to lysosomes [24]. The recognition of newly synthesized pCD by phosphotransferase in the cis-Golgi, resulting in M6P-labelling of oligosaccharides and subsequent capture by M6P receptors, is the predominant mode of lysosomal targetting. An alternative pathway has also been shown to operate in cells incapable of M6P labelling [24,25]. In addition to the two proposed pathways of lysosomal targeting, there are several reports of M6P-independent association of pCD with various intracellular membranes [26–29]. It was suggested that the propeptides of lysosomal proteinases might be responsible for these interactions [17]. Grassel and Hasilik [30] identified a 60 kDa protein which is specifically associated with the pCD molecule. A role for the propeptide in the M6P-independent association of procathepsin L with intracellular membranes was shown [26,31,32]. The propeptide of procathepsin L was also suggested to promote the proliferation of immature thymocytes [33]. The involvement of the propeptides of aspartic vacuolar proteinase A from Saccharomyces cerevisiae in targeting to acidic vacuoles has been demonstrated [34]. These data support the concept that the propeptides of lysosomal proteinases have biologically significant functions other than only acting to inhibit lysosomal enzymes before they reach the lysosome.

Our present results show that the propeptide of human pCD plays an important role in the mitogenic function of pCD. Inhibition of this function by propeptide-specific antibodies and comparable activity of a synthetic propeptide indicated the existence of an interaction between the propeptide and a surface receptor. This conclusion was supported by experiments on pCD association with cell surfaces, and inhibition of this association by the addition of the propeptide. The mitogenic effect of this peptide, when used in 50-fold higher molar concentration (20 nM), was very similar to that of isolated pCD (the concentrations of pCD found in supernatants of cell line ZR-75-1 treated with oestrogen are comparable with the concentrations of pCD used in our study [13]). There was no activity detected when a peptide of the same size but with a scrambled sequence was used (result not shown). Partial lower activity of the propeptide of pCD in comparison with intact pCD may be explained by its higher conformational flexibility compared with the structure of the same region of pCD, or may be a result of its only forming part of a three-dimensional signal present on the entire pCD structure.

In conclusion we propose that the mechanism of the mitogenic function of pCD relies on a specific structure of the propeptide of pCD, and its interaction with a cell-surface-localized receptor. Our findings identify the propeptide of pCD as a new potential target for suppression of growth of certain types of breast tumours.

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